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The SUMO Pathway Promotes Basic Helix-Loop-Helix Proneural Factor Activity via a Direct Effect on the Zn Finger Protein Senseless

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During development, proneural transcription factors of the basic helix-loop-helix (bHLH) family are required to commit cells to a neural fate. In *Drosophila* neurogenesis, a key mechanism promoting sense organ precursor (SOP) fate is the synergy between proneural factors and their coactivator Senseless in transcriptional activation of target genes. Here we present evidence that posttranslational modification by SUMO enhances this synergy via an effect on Senseless protein. We show that Senseless is a direct target for SUMO modification and that mutagenesis of a predicted SUMOylation motif in Senseless reduces Senseless/proneural synergy both *in vivo* and in cell culture. We propose that SUMOylation of Senseless via lysine 509 promotes its synergy with proneural proteins during transcriptional activation and hence regulates an important step in neurogenesis leading to the formation and maturation of the SOPs.

Cell fate decisions in neurogenesis require proneural proteins, which are basic helix-loop-helix (bHLH) transcription factors (7). In *Drosophila*, proneural proteins including Achaete (AC), Scute (SC), Atonal (ATO), and Amos (AMOS) are essential for the specification of sense organ precursors (SOPs) for the various sense organs of the peripheral nervous system. The proneural proteins are first expressed at low levels in groups of uncommitted ectodermal cells known as the proneural clusters. This initial expression is under the control of upstream prepattern transcription factors, such as Pannier and Iroquois (42, 50). Subsequently, in some cells (the presumptive SOPs) proneural proteins promote upregulation of their own expression via autoregulatory enhancers. The proneural proteins bind to E-boxes within these enhancers as heterodimers with the ubiquitously expressed bHLH protein Daughterless (DA). The transition to autoregulation appears to be critical for neural commitment and downstream gene activation (15, 69). How this transition is controlled is therefore an important question. One of the factors regulating the specification process is lateral inhibition via the Notch signaling pathway (4, 33). This counteracts proneural gene upregulation via the autoregulatory enhancers in non-SOP cells through the inhibitory Enhancer of split [E(SPL)] bHLH proteins.

Another factor affecting proneural activity is the Zn finger transcription factor, Senseless (SENS). SENS belongs to the GPS (Gfi1/Pag-3/Senseless) family of proteins (30). This factor is crucial for the production of mature SOPs (47, 53). In *sens* mutants, SOPs begin to form but fail to complete specification, instead undergoing apoptosis (47). SENS's role in promoting neurogenesis appears to be closely involved with modulating proneural factor activity. Without SENS the SOPS fail to maintain proneural protein levels required for formation and survival (47, 53). *sens* is a direct proneural target gene (29), and subsequently SENS protein is coexpressed with proneural proteins in SOPs (29, 70). In this context SENS enhances proneural factor activity as a transcriptional coactivator: it can form complexes with AC/DA and SC/DA that greatly enhance their ability to stimulate gene expression via E-box binding sites (3, 29, 49). Although originally shown for AC and SC (3), the same coactivation synergy can also be observed for ATO/DA/SENS and AMOS/DA/SENS (49) and so appears to be a general mechanism of proneural factor modulation. Proneural/SENS synergy is thought to have an important role in bypassing the negative regulatory effects of Notch signaling in the SOP.

In addition to this coactivator activity, SENS can act as a direct transcriptional repressor. This is mediated by SENS binding directly to an S-box motif (29, 43). In this manner, SENS represses the *rhomboid* (*rho*) gene in embryonic abdominal segments (65). Interestingly, repression via an S box has also been shown for the *ac* autoregulatory enhancer (29). Thus, SENS can both repress and promote *ac* transcription: binding to the S box directly inhibits proneural autoregulation, whereas binding to proneural factors promotes autoregulation via the E box (15, 29). This contradictory function led to the proposal that SENS acts as a binary switch to promote either proneural protein activation or proneural gene repression under different circumstances (Fig. 1A and B) (29). In this model, manipulation of SENS activity could have a profound effect on proneural function and SOP formation, but it is unclear how the switch between activating and repressing modes of SENS action might be regulated. The initial suggestion was that it depends on the level of SENS expression, such that low levels are repressive (in ectodermal cells that will not become SOPs) while coactivation is triggered only at higher expression levels (in SOPs) (29). In this paper we address the possibility that posttranslational modification might influence SENS's function. Specifically, we present evidence that modification by small ubiquitin-related modifier (SUMO) protein promotes SENS's coactivator activity.

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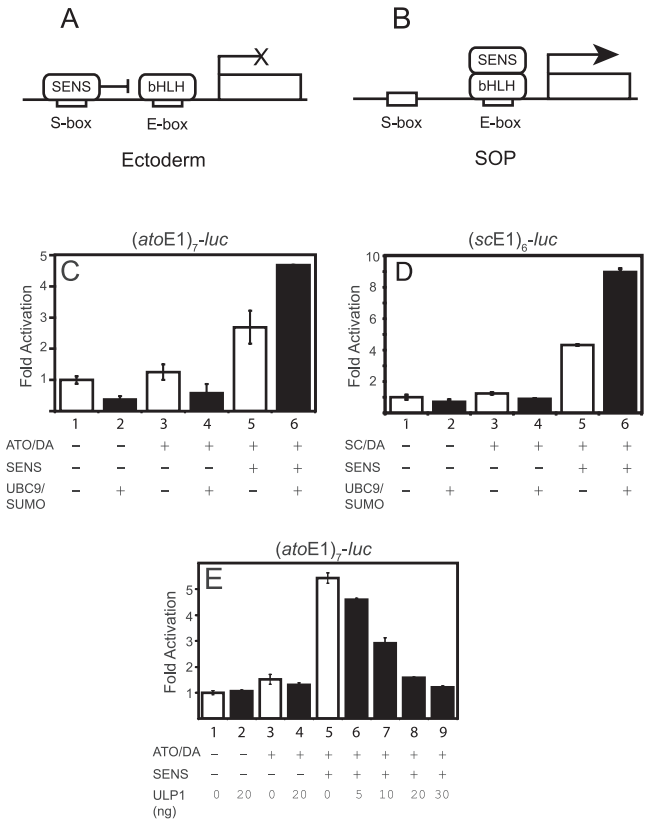

FIG 1 SUMO enhances the transcriptional synergy between SENS and proneural proteins in S2 cells. (A, B) Schematic illustration of the binary switch model for proneural gene transcriptional regulation by SENS (29). (A) In the ectodermal cells surrounding the SOP, low levels of SENS promote DNA-binding-dependent repression of proneural gene transcriptional activation by proneural-DA heterodimers (bHLH). (B) In the SOP, higher levels of SENS synergize with the proneural proteins to enhance proneural gene transcriptional activation independent of DNA binding by SENS. (C, D, and E) Luciferase assays for ATO (C and E) and SC (D) reporter genes in S2 cells. Note that the E-box reporter constructs used for these three panels lack SENS binding sites. (C and D) Cotransfection of plasmids expressing SUMO and UBC9 (black bars) has a repressive effect on proneural reporter gene transcriptional activation regardless of the presence of proneural proteins (bars 1 to 4). However, SUMO/UBC9 enhances transcriptional activation driven by proneural-SENS complexes (column 6 compared with column 5). (E) The ULP1 SUMO protease (black bars) promotes a dose-dependent decrease in transcriptional synergy of the ternary ATO/DA/SENS complex (bars 6 to 9 compared with bar 5). In the absence of SENS, ULP1 has no effect on transcriptional activation (bars 2 and 4 versus 1 and 3, respectively).

SUMO proteins are ubiquitin-like proteins that can become conjugated to lysine residues in target proteins (27, 28). SUMO and ubiquitin have similar structures, and their conjugation mechanisms are remarkably similar. However, the function of SUMOylation is quite distinct from that of ubiquitination. SUMO targets are mostly high-molecular-weight nuclear proteins, and SUMOylation alters their cellular function in a variety of ways, including prevention of degradation by way of competing for ubiquitination sites (26), alteration of subcellular localization (9), modulation of protein stability (6), and modification of transcription factor activity (19, 21, 62). SUMO modification consequently has an important role in a large variety of biological processes.

Here we present data from *in vivo* and tissue culture experiments consistent with a role for SUMOylation in promoting the proneural/SENS synergy in *Drosophila*. In an S2 cell luciferase assay, transcriptional activation synergy between SENS and the proneural proteins SC and ATO was increased by enhancing SUMOylation and decreased by deSUMOylation. Consistent with a direct role for SUMO, SENS is SUMO-modified in *Drosophila* S2 cells and in an *in vitro* SUMOylation assay, and SENS with a mutated SUMOylation motif was refractory to SUMO stimulation. An *in vivo* misexpression assay revealed genetic interactions between SUMO and SENS, as well as with the proneural proteins themselves, that were dependent on the presence of a SUMOylation motif. We propose that SUMOylation of SENS via lysine 509 (K509) promotes its synergistic interaction with proneural proteins, thereby regulating their activity, and hence regulating an important step in neurogenesis.

MATERIALS AND METHODS

Fly stocks. Fly stocks used as previously described are 109(2)68*gal4*/*CyO* (31), UAS-*ato* (32), UAS-*sc* (13), and UAS-*sens* (47). UAS-*FLAG-sumo*/*CyO* flies were a kind gift from Minghua Nie and Albert Courey. UAS-*sens*-attB lines for wild-type and mutated SENS were made as described below.

Plasmid constructs. Protein expression constructs RactHAdh, RactH-Adh-*da*, and pAc-*sc* were donated by Christos Delidakis (20). pAc-*sens* for SENS expression in the S2 cell cotransfection assay was a kind gift from Hamed Jafar-Nejad (29). pAc-*FLAG-sens* for expression of SENS with an N-terminal FLAG tag was made by inserting the following oligonucleotide in the *Asp*718 sequence preceding the ATG in pAc-*sens*: FLAG top, GTA CCAAAATGGACTACAAGGACGACGATGACAAGC; FLAG bottom, GTACGCTTGTCATCGTCGTCCTTGTAGTCCATTTTG.

The SENS coding sequence was cloned in the mammalian expression vector pEGFP-C1 (Clontech) for transfection in HeLa cells and expression of a GFP-SENS fusion protein. The following primers were used to amplify the *sens* sequence by PCR using pAc-*sens* as the template. The restriction sites for SalI and XmaI used in cloning are underlined, and the ATG start codon and TGA stop (TCA on reverse strand) are in bold: Sens forward, ACGCGTCGAC**ATG**AATCACCTATCGCCG; Sens reverse, TA AACCCGGG**TCA**GCAGCTGCTGCTGCTCAC.

Site-directed mutagenesis of lysine to arginine for the predicted SUMOylation motifs in SENS p-EGFP-C1-*sens* (see Table 2) was carried out using the QuikChange mutagenesis kit from Agilent Technologies and the primers below, except for K533R, which was PCR amplified as a linear fragment with the mutation in the 3′ primer. Primers (with the codon encoding arginine indicated in boldface) included (i) K229R forward, TACGGCCTG**AGG**ATGGAGGAG, and reverse, CTCCTCCAT**CCT**CA GGCCGTA; (ii) K249R forward, GTGCGAAAGTTC**AGG**TATGAGCGC AGAACTGC, and reverse, GCAGTTCTGCGCTCATA**CCT**GAACTTTC GCCAC; (iii) K313R forward, GCACTTGAAGCTG**AGG**AGCGAGCAG CCGC, and reverse, GCGGCTGCTCGCT**CCT**CAGCTTCAAGTGC; (iv) K327R forward, CATCGCATC**AGG**GACGAGCAG, and reverse, CTGC TCGTC**CCT**GATGCGATG; (v) K533R forward, ACGCGTCGACATG AATCACCTATCGCCG, and reverse, TAAACCCGGGTCAGCAGCTGC TGCTGCTCACCTCCAT**CCT**CA; (vi) K476R forward, GTGTGCCTG**A GG**GCCTTCAGC, and reverse, GCTGAAGGC**CCT**CAGGCACAC; (vii) K453R forward, TTCCACCAA**AGG**TCGGACATG, and reverse, CATGT CCGA**CCT**TTGGTGGAA; (viii) K91R forward, ATGACCCCC**AGA**TCG CCCGCC, and reverse, GGCGGGCGA**TCT**GGGGGTCAT; (ix) K509R forward, TTCCAGCGC**AGG**GTGGATCTG, and reverse, CAGATCCAC **CCT**GCGCTGGAA; (x) K448R forward, TATTGTGGC**AGG**CGGTT CCAC, and reverse, GTGGAACCG**CCT**GCCACAATA; (xi) K420R forward, CAGTGTGGC**AGG**AGCTTCAAG, and reverse, CTTGAAGCT **CCT**GCCACACTG; and (xii) D511A forward, CAGCGCAAGGTG**GCT** CTGCGACGTCAT, and reverse, GTCGCGTTCCAC**CGA**GACGCTGC AGTA.

The RKVD SUMO motif at amino acids 510 to 512 within the SENS sequence of pAc-*FLAG-sens* was mutated to give SENS^{K509R} and

SENS^{D511A} variants for use in the S2 cell luciferase assay using the QuikChange II XL mutagenesis kit from Agilent Technologies and the following primers: for SENS^{K509R}, SensA1744G forward, GTCCTTCCAG CGCAGGGTGGATCTGCG, and SensA1744G reverse, CGCAGATCCA CCCTGCGCTGGAAGGAC; for SENS^{D511A}, Sens A1750C forward, CCA GCGCAAGGTGGCTCTGCGACGTCATC, and SensA1750C reverse, GATGACGTCGCAGAGCCACCTTGCGCTGG.

For Western blot analysis, a construct for expression of SENS with a 3× FLAG N-terminal tag (Sigma), under the control of the Actin5C promoter, was made by Gateway cloning (Invitrogen) of the SENS coding sequence into the vector pAFW. pAFW was a kind gift from Terence Murphy, Carnegie Institution of Washington.

pUASTattB vector for germ line transformation (see below) using the phiC31 integrase was a kind gift from J. Bischof (10). For pUASTattB constructs the coding sequences for wild-type SENS, SENS^{K509R}, and SENS^{D511A} were amplified from the relevant pAc-*FLAG-sens* constructs by PCR using the following primers (with restriction sites in bold) and the Roche Expand Long Template PCR system: SensNotI forward, GAC**GCG GCCGC**AAAATGAATCACCTATCGCCGCCGCC; SensXbaI reverse, GAC**TCTAGA**GGATCAGAGATTGCCGCCTAGCCTCG.

The PCR products were TA cloned using the Strataclone PCR cloning kit (Agilent Technologies), their sequences were checked using the sequencing primers described below, and then they were cloned in pUASTattB using the NotI and XbaI restriction sites. The sequencing primers used were pUASTattB forward, CAACTGCAACTACTGAAATC TGCC, and reverse, CACACCACAGAAGTAAGGTTCC.

S2 cell cotransfection luciferase assays. S2 cell cotransfection assays were carried out using the Promega DLR system as described previously (49). Each cotransfection was done in triplicate, and the data in each experiment were normalized to a zero expression construct data set (i.e., cells transfected with empty vector). Concatemerized E-box luciferase reporter constructs were as described in reference 49. For pGL4-AcP the 455-bp *achaete* promoter fragment made by PCR from genomic DNA using the following primers was cloned in pGL*4-luc* using *Asp*718 and HindIII: Ac forward, GATCGGTACC GGATGGCCACTTTCAATAGGAG, and Ac reverse, GCGCAAGCTTCG CTGCCCAAAGCCATTTTAAG.

SUMO system expression constructs, kindly donated by A. Courey, included pPAC-HA-Ubc9, pPAC-HA-Smt3, pPac-HA-DmSAE1, and pPac-HA-Dm-SAE2 (9) and pPAC-FLAG-Ulp1 (8, 57). Cells were incubated for 24 h at 27°C posttransfection and pelleted, and lysates were made using the Promega passive lysis buffer. For low proneural concentration for luciferase assays, 0.5 ng proneural expressing plasmids was transfected per 1 ml of cells at 0.5×10^6 cells/ml, while for high proneural concentration the amount of expression construct was 20 ng. SENS expression plasmid was always at 20 ng per ml of cells. For Western blot analysis, expression plasmid per ml of cells was for SENS at 50 ng, for SUMO at 12.5 to 50 ng, for ULP1 at 50 to 100 ng, and for UBC9 at 50 ng. Western blot lysates were processed in the presence or absence of 20 mM *N*-ethylmaleimide (NEM) (Sigma-Aldrich) as indicated. Lysate (40%; 20 μl from 50 μl lysate per 1 ml of cells) was loaded in each case onto an 8% SDS-PAGE gel, followed by Western blotting. Protein was visualized using monoclonal mouse anti-FLAG antibody (Sigma).

HeLa cell cotransfection localization assay. HeLa cells were maintained and transfected as described previously (12). Constructs used for transfection were RFP-SUMO-1 and RFP-SUMO-2 (12) and wild-type and mutated pEGFP-C1-SENS as described above. The cells were incubated for 12 h at 37°C before analysis by fluorescence microscopy.

Germ line transformation. Transgenic *Drosophila* lines were made using the phiC31 integrase system to insert SENS plasmids into an attP2 landing site on chromosome 3L (10, 23). Insertion at the same landing site allowed quantitative comparison of their effects on neurogenesis. pUAST-SENS-attB wild-type and mutant constructs were injected into embryos heterozygous for the attP2 landing site (23) and for the phiC31 integrase driven by the *nanos* promoter, located on the X chromosome (Genetic Services Inc., Sudbury, MA). Male flies that developed from injected embryos were crossed to w^{1118} virgin females, and transformants were selected from the offspring based on the presence of the red eye color due to expression of the vector-borne *w* gene. Red-eyed males were selected (ensuring loss of the integrase) and crossed to *yw*; *Ly/TM3*, *Sb*. Transgenic stocks were made by selecting red-eyed Sb males and females and crossing them together to allow selection of non-*Sb* homozygous flies.

Yeast two-hybrid analysis. Yeast two-hybrid analysis employed the Matchmaker Two-Hybrid system (Clontech). PCR was used to amplify the coding sequences of SUMO^{ACT} (an activated form of *Drosophila* SUMO lacking the 4 C-terminal residues, revealing the C-terminal diglycine) and SUMOΔGG (nonconjugatable SUMO) from pPAC-smt3 (9) for cloning in the yeast two-hybrid pAS2-1 bait vector, using the SfiI and XmaI sites to make a construct encoding a fusion of the relevant SUMO variant with the yeast GAL4 DNA binding domain (BD). The primers used were as follows: pAS2-1-Smt3 forward primer (the SfiI site is in italics and the ATG start methionine in bold; used for both dSmt3-GG and dSmt3-ΔGG), TATA*GGCCATGGAGGCC***ATG**TCTGACGAAAAGAAG; pAS2-1-Smt3-GG reverse primer (XmaI site in italics), ATAT*CCCGGG*T TAGCCACCAGTCTGCTGCTG; pAS2-1-Smt3-ΔGG reverse primer (XmaI site in italics), ATAT*CCCGGG*TTAAGTCTGCTGCTGGTAAAC.

SENS wild-type, SENS^{K509R}, and SENS^{D511A} sequences were cloned in the SfiI/XmaI sites of the prey plasmid pACT2 following PCR from the appropriate pEGFP-SENS variant construct using the following primers to make constructs encoding fusions of the yeast GAL4 activation domain with the relevant SENS variant: pACT2 SENS forward primer (the SfiI site is shown in italics and ATG in bold), TATA*GGCCATGGAGGCC***ATG**AA TCACCTATCGCCG; and pACT2 SENS reverse primer (the XmaI site is shown in italics and STOP [TCA] in bold), ATAT*CCCGGG***TCA**GCAGC TGCTGCTGCTCAC.

Transformations of the CG1945 strain and plating on selective plates were performed according to the manufacturer's protocol. 5-Fluoroorotic acid was from Stratech Scientific. Amino acids and 3-aminothymine were from Sigma, yeast extract-peptone-dextrose (YPD) medium was from Clontech, and Difco yeast nitrogen base, Bacto peptone, and Bacto yeast extract were from Becton Dickinson.

***In vitro* SUMO conjugation assays.** *In vitro* SUMO conjugation assays were carried out according to the method described in reference 12 for N-terminally glutathione *S*-transferase (GST)-tagged 20-amino-acid SENS fragments (GST-SENS-f) corresponding to amino acids 500 to 519 of the full-length SENS for wild-type, K509R, and D511A SENS variants. The reaction mixture contained 0.5 μg GST, 2 μg GST-SENS-f, 0.5 μg GST-D4* (a DAXX-4 fragment, which served as a positive control), 2 μg His-SUMO, 0.1 μg recombinant GST-SAE1/2 (E1), 0.1 μg GST-Ubc9 (E2), and 10 μl ATP regenerating system (50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 mM ATP, 10 mM creatine phosphate, 3.5 U/ml creatine kinase, and 0.6 U/ml inorganic pyrophosphatase). After incubation at 37°C for 3 h, the mixture was stopped by adding sample buffer, boiled, and then separated by 12.5% SDS-PAGE, and SUMOylation substrates and conjugates were visualized by blotting with rabbit anti-GST antibodies. The SUMOylation conjugates were confirmed by blotting the stripped membranes with rabbit anti-His antibodies.

RESULTS

SUMO promotes transcriptional activation by the proneural/SENS ternary complex in S2 cells. The E-box-dependent transcriptional activation synergy between proneural proteins and SENS can be observed in an S2 cell luciferase reporter gene assay using both a construct based on an autoregulatory enhancer from the *ac* gene (*acP-luc*) (3, 29) and concatemerized E-box reporter constructs (*sc-luc* and *ato-luc*) (49) (Fig. 1). At low plasmid levels, transfection of either ATO/DA or SC/DA does not result in significant activation of the concatemerized E-box luciferase reporter constructs (Fig. 1C and D, column 3). SENS transfected alone has no effect on transcriptional activation, even at high concentra-

tions (49). However, cotransfection of SENS and proneural/DA results in significant activation (49) (Fig. 1C and D; compare bars 5 and 3 in each panel). This effect is thought to occur via the formation of a ternary proneural/SENS complex. It is E-box dependent but does not require the DNA-binding ability of SENS (3, 29, 49) (Fig. 1). It has previously been shown that *Drosophila* SUMO substrates can be SUMO modified in S2 cells both by endogenous SUMO and by SUMO expressed from a cotransfected expression construct (9). We therefore used this assay to test the effect of SUMO components on proneural/SENS activity.

When constructs expressing components of the SUMOylation pathway (UBC9 [the SUMO conjugating enzyme] and Smt3 [SUMO]) are transfected into S2 cells, there is a strong downregulation of background reporter gene activity (Fig. 1C and D; see bar 2 in each panel), which is perhaps consistent with SUMO's general role in repression of transcription; for example, by histone deacetylase (HDAC) recruitment (18). This general repressive effect of SUMOylation is still evident in the presence of SC/DA or ATO/DA (Fig. 1C and D; bar 4 compared to bar 3 in each case). In contrast, however, SUMO promotes a dramatic increase in SC/DA/SENS and ATO/DA/SENS activation of reporter genes (Fig. 1C and D, bar 6 versus bar 5). This observation is consistent with a role for SUMOylation in specifically promoting the formation, function, or stability of the proneural/SENS complex.

SUMO modification is a transient process, and the SUMO moiety can be removed from a modified protein by SUMO proteases. In common with yeast and mammals, the *Ulp1* cysteine protease performs de-SUMOylation in *Drosophila* (8, 57). In the reporter gene assay, cotransfection with increasing amounts of *Ulp1*-expressing plasmid caused a corresponding decrease in ATO/DA/SENS-dependent reporter gene activation for *ato-luc* (Fig. 1E), *sc-luc*, and *ac*P-*luc* (data not shown). This occurred in the absence of cotransfected SUMO/UBC9, consistent with an effect via endogenous SUMO pathway components within the S2 cells. We conclude that proneural/SENS activity is enhanced by SUMOylation, and indeed it is largely dependent on endogenous SUMO activity in S2 cells. Furthermore, the effect of SUMOylation is seen only in the presence of SENS, consistent with SENS being the target of SUMOylation.

SENS interacts with SUMO in a yeast two-hybrid assay and is SUMOylated in S2 cells. The effect of SUMO in the S2 reporter assay suggests an effect via SENS itself. For other SUMO substrates, such as the chromosomal passenger complex protein, Borealin, and the Daxx transcriptional repressor, interaction with SUMO has been demonstrated by a yeast two-hybrid assay (12, 36), so we used the same approach to test whether SENS and SUMO interact. First, we tested the interaction of SENS with the activated form of SUMO ($SUMO^{ACT}$), which lacks the two C-terminal amino acids of full-length SUMO revealing the C-terminal diglycine (12). Our yeast two-hybrid results indicated a clear interaction between SENS and $SUMO^{ACT}$, supporting the possibility that SENS is a substrate for SUMOylation.

We next used the yeast two-hybrid assay to determine whether SENS also interacts with a nonconjugatable form of SUMO ($SUMO^{\Delta GG}$) that lacks 6 C-terminal amino acids including the C-terminal diglycine (37). An interaction was indeed observed between SENS and $SUMO^{\Delta GG}$ (Table 1), suggesting noncovalent interaction between the two, but this was weaker than that observed with $SUMO^{ACT}$. One possible reason for the weaker interaction with $SUMO^{\Delta GG}$ is that, in contrast to $SUMO^{ACT}$, the nonconjugatable form has a mostly cytoplasmic rather than nuclear location (12). In addition to covalent modification, many SUMO substrates also interact noncovalently with SUMO via hydrophobic regions, and such interactions have been found to be important for subsequent covalent modification (44, 58). The interaction of SENS with both $SUMO^{ACT}$ and $SUMO^{\Delta GG}$ therefore implies that SENS is a strong candidate for SUMO modification.

TABLE 1 Yeast two-hybrid results demonstrating interactions between SENS and SUMO[a]

Protein	BD-vector	BD-$SUMO^{ACT}$	BD-$SUMO^{\Delta GG}$
AD-DAXX[b]	ND	+ + +	ND
AD-SENS	−	+ + +	+
AD-$SENS^{K509R}$	−	+ +	+
AD-$SENS^{D511A}$	−	+ +	+

[a] The interactions were confirmed by the presence of colonies on SC-Leu-Trp-His selective plates (HIS reporter gene) and also by positive β-galactosidase assays. AD and BD, GAL4 activation and DNA-binding domains, respectively, in the yeast two-hybrid prey and bait constructs; ND, not done.
[b] Positive control.

In order to investigate whether SENS is covalently modified by SUMO, we transfected *Drosophila* S2 cells with a FLAG-tagged SENS expression construct and various combinations of plasmids expressing components of the SUMOylation machinery. Western blotting with an anti-FLAG antibody was then used to analyze the lysates (Fig. 2A and B). FLAG-SENS was detected as a strong band migrating at 100 kDa (Fig. 2A, arrow). An additional higher-molecular-weight band (Fig. 2A, asterisk) was observed. This band was strongly dependent on the presence of the SUMO protease inhibitor, *N*-ethylmaleimide (NEM) (Fig. 2B). Moreover, it disappeared upon cotransfection with the SUMO protease ULP1 (Fig. 2A, lane 5). Hence, we conclude that the extra band represents SUMOylated SENS protein. Interestingly, the additional band could be observed both in the presence of exogenous SUMO and/or Ubc9 and with SENS alone, consistent with the conclusion above stating that SENS activity is modified by endogenous SUMOylation activity. Additional higher-molecular-weight bands may correspond to minor species with more than one lysine SUMOylated (14).

In HeLa cells SENS localizes to PML bodies in a SUMO-dependent manner. To look for further evidence of an interaction between SENS and SUMO, we analyzed their subcellular distribution in cultured cells. In mammalian cells, SUMO is localized to punctate subnuclear structures called promyelocytic leukemia protein (PML) nuclear bodies. Interaction between transcription factors and SUMO can alter their localization to these PML bodies (11, 12, 38, 44). For example, SUMO-1 modification of the zinc finger protein ZNF198 has been shown to localize it to the PML nuclear bodies of Hek 293 cells (38). Less-distinct punctate staining is also found in *Drosophila* nuclei, but its relationship with PML bodies is not clear (41, 57). Hence, we used localization in HeLa cells to investigate whether SENS and SUMO are capable of interacting.

We used GFP-tagged SENS and human RFP-SUMO-1 and RFP-SUMO-2 to test whether SENS and SUMO colocalize in HeLa cells (Fig. 3). Human SUMO-1 and SUMO-2 show 52% and 71% identity, respectively, to *Drosophila* SUMO (52). SUMO-1 or SUMO-2 localized to the PML bodies of the cell, as shown by costaining with PML antibody (results not shown) (68). When GFP-SENS was transfected alone, it localized to the nucleus in a

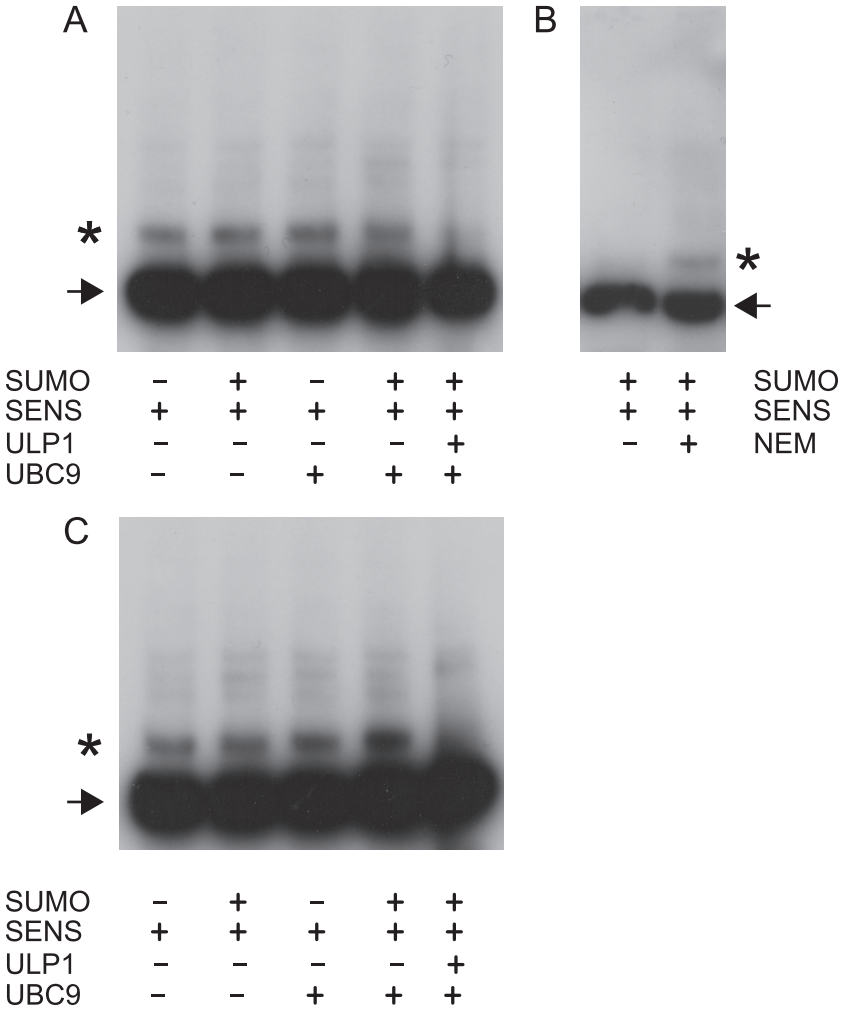

FIG 2 SENS is SUMOylated in S2 cells. (A and B) Western blots with anti-FLAG antibody of S2 cell lysates after transfection with plasmids for FLAG-SENS, SUMO, ULP1, or UBC9 as indicated. (A) Anti-FLAG antibody detects unmodified FLAG-SENS (arrow) and an additional lower-mobility band proposed to be SUMOylated SENS (asterisk). This SUMO-SENS band is observed when SENS is transfected alone (lane 1) or is cotransfected with plasmids expressing SUMO (lane 2), UBC9 (lane 3), or both SUMO and UBC9 (lane 4), but it is absent when the SUMO protease Ulp1 is cotransfected (lane 5). The presence of the SUMO-SENS band in the absence of cotransfected SUMO (lane 1) suggests SENS modification by endogenous SUMO. (B) The SUMO-SENS band is dependent on the presence of the SUMO protease inhibitor, NEM, in the lysates (lane 2 compared to lane 1). Note that NEM is present in all lysates in panel A. (C) Western blot with anti-FLAG antibody of S2 cell lysates after transfection with plasmids for FLAG-SENS^{D511A}, SUMO, ULP1, or UBC9 as indicated. SUMOylated SENS (asterisk) was detected as for the wild-type protein.

diffuse pattern excluding the nucleoli (Fig. 3A). When GFP-SENS and RFP-SUMO-1 or 2 were cotransfected, the SENS protein became dramatically redistributed so that it colocalized with the SUMO in the PML bodies (Fig. 3B and C). This is consistent with a direct interaction between SENS and SUMO in HeLa cells.

Lysine at position 509 is important for SUMO-dependent SENS relocalization in HeLa cells. As the HeLa cell results are consistent with a direct interaction between SUMO and SENS, we investigated the effects of mutating potential SUMO target lysines on the localization of SENS. Typically SUMO is conjugated to lysine residues in the consensus sequence ΨKxD/E (where Ψ is a large hydrophobic residue and x is any residue) (51), although not all such motifs will be SUMOylated and nonconsensus SUMOylation sites are also known (27). Analysis of the SENS sequence using SUMOplot (Abgent) identified 11 potential sites for SUMO modification, five of which are predicted with high probability (Fig. 3D). We investigated the importance of these motifs in the HeLa cell localization assay. Lysines in the predicted SUMO target sequences were mutated to arginine to prevent SUMO conjugation, either individually or in combination (Table 2), and the subnuclear localization in HeLa cells was determined for each of these mutants. This showed that up to 8 of the 11 motifs could be mutated in combination (SENS-m8 construct; Fig. 4B) with no disruption of the localization of the SENS protein and no change in its relocalization with SUMO upon cotransfection (Fig. 4A and C). In contrast, mutation of one additional lysine (K509) resulted in disruption of the subnuclear localization (construct SENS-m9; Fig. 4E). Sens-m9 transfected alone was seen in nuclear dots, distinct from the PML bodies (Fig. 4D), rather than in the diffuse pattern seen for wild-type protein. Moreover, SENS-m9 did not relocate to PML bodies upon SUMO cotransfection (Fig. 4F). We then examined the effect of mutating K509 alone. Mutation

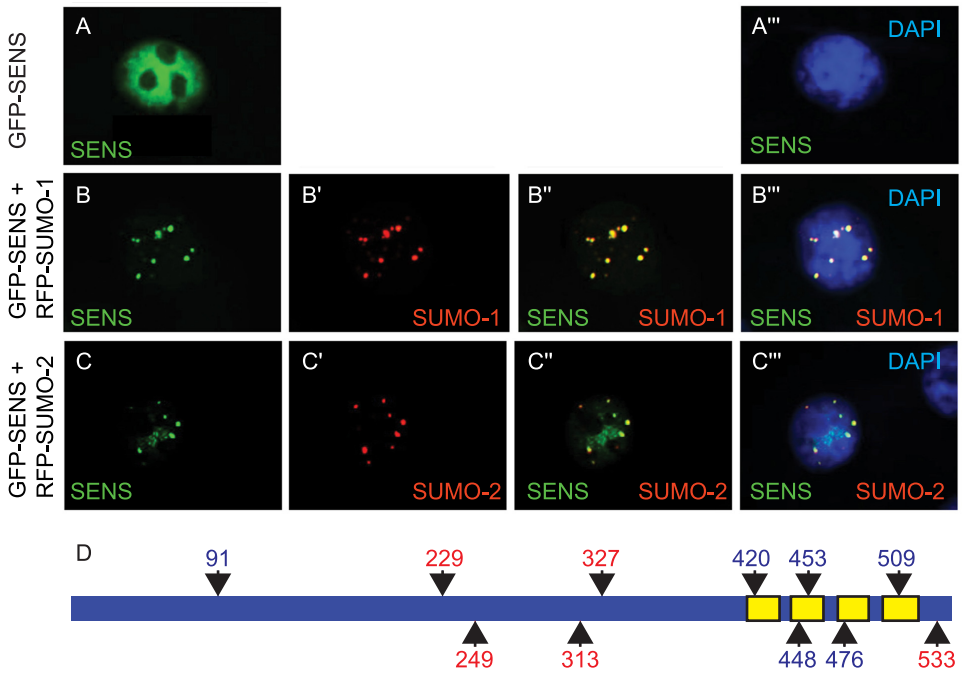

FIG 3 SENS is relocalized to PML bodies in HeLa cells in a SUMO-dependent manner. (A) GFP-SENS transfected into HeLa cells localized diffusely to the nucleus as indicated by costaining with DAPI (4′,6-diamidino-2-phenylindole) (A‴). (B and C) GFP-SENS cotransfected with human SUMO-1 (B to B‴) or SUMO-2 (C to C‴) redistributed in a punctate pattern showing colocalization with SUMO-1 or SUMO-2. (D) Schematic diagram of SENS (541 amino acids in length) showing the four C-terminal Zn fingers (yellow) and the 11 SUMOylation motifs predicted by SUMOplot (Abgent). Higher-probability sites are shown in red and lower-probability sites in blue.

of K509 (the SENS^{K509R} mutant construct) resulted in alteration of the subnuclear localization of SENS in a proportion (59.3% ± 2.7%) of the transfected cells (Fig. 4G and H). In addition, SENS^{K509R} failed to colocalize with SUMO in most cells (Fig. 4I and J; no colocalization in 95.3% of cells). We conclude that K509 is important for SUMO-dependent localization of SENS and may be a major site of SUMOylation, although other sites may contribute as well.

As lysine residues can be subject to various posttranslational modifications, including acetylation and ubiquitination, we confirmed a SUMO-specific effect of K509 by mutating the third residue in the SUMOylation motif to give SENS^{D511A}. The third residue is specific for UBC9 interactions and would not affect other modifications of the lysine residue in the motif (45). SENS^{D511A} failed to colocalize with SUMO in all cells (data not shown), consistent with mutation of the motif disrupting a covalent interaction with SUMO.

TABLE 2 Summary of the SENS SUMO motif mutations

Mutant name	Lysine(s) mutated
Sens wild type	None
Sens-327-mut	K327
Sens-229-mut	K229
Sens-533-mut	K533
Sens-313-mut	K313
Sens-249-mut	K249
Sens-M2	K327, K533
Sens-M3	K327, 313, 533
Sens-M4	K327, 313, 533, 249
Sens-M5	K327, 313, 533, 249, 229
Sens-M6	K327, 313, 533, 249, 229, 476
Sens-M7	K327, 313, 533, 249, 229, 476, 453
Sens-M8	K327, 313, 533, 249, 229, 476, 453, 91
Sens-M9	K327, 313, 533, 249, 229, 476, 453, 91, 509
Sens-M10	K327, 313, 533, 249, 229, 476, 453, 91, 509, 448
Sens-M11	K327, 313, 533, 249, 229, 476, 453, 91, 509, 448, 420
Sens-509-mut	K509

In contrast with the colocalization assay results, both SENS^{D511A} and SENS^{K509R} retained the ability to interact with SUMO in the yeast two-hybrid assay (Table 1) consistent with the presence of additional SUMO target lysines. For both SENS^{D511A} and SENS^{K509R}, interaction with SUMO^{ACT} was, however, weaker than for wild-type SENS, consistent with an important role for this SENS SUMOylation motif in the SUMO-SENS interaction.

SENS SUMOylation mutants are refractory to SUMO modification in vitro but not in S2 cells, suggesting SUMOylation at an alternative site or sites in S2 cells. In order to test the ability of the putative SUMO acceptor K509 to be SUMOylated, we used an *in vitro* SUMOylation assay (12, 60). GST-tagged 20-amino-acid peptides containing the K509 SUMO motif and surrounding amino acids were used to assess the efficiency of SUMOylation of wild-type, K509R, and D511A versions of the motif. In this assay only the wild-type sequence was SUMO modified, demonstrating that K509 is indeed an acceptor for SUMOylation and that both mutations prevent this SUMOylation (Fig. 5A and B).

We then tested the effects of the mutations in the context of full-length SENS in S2 lysates. In this case SUMO-modified SENS was still seen for both SENS^{D511A} (Fig. 2C) and SENS^{K509R} (data not shown). We suggest that this may arise due to SUMOylation at one or more alternative lysines in the SENS sequence, as has been

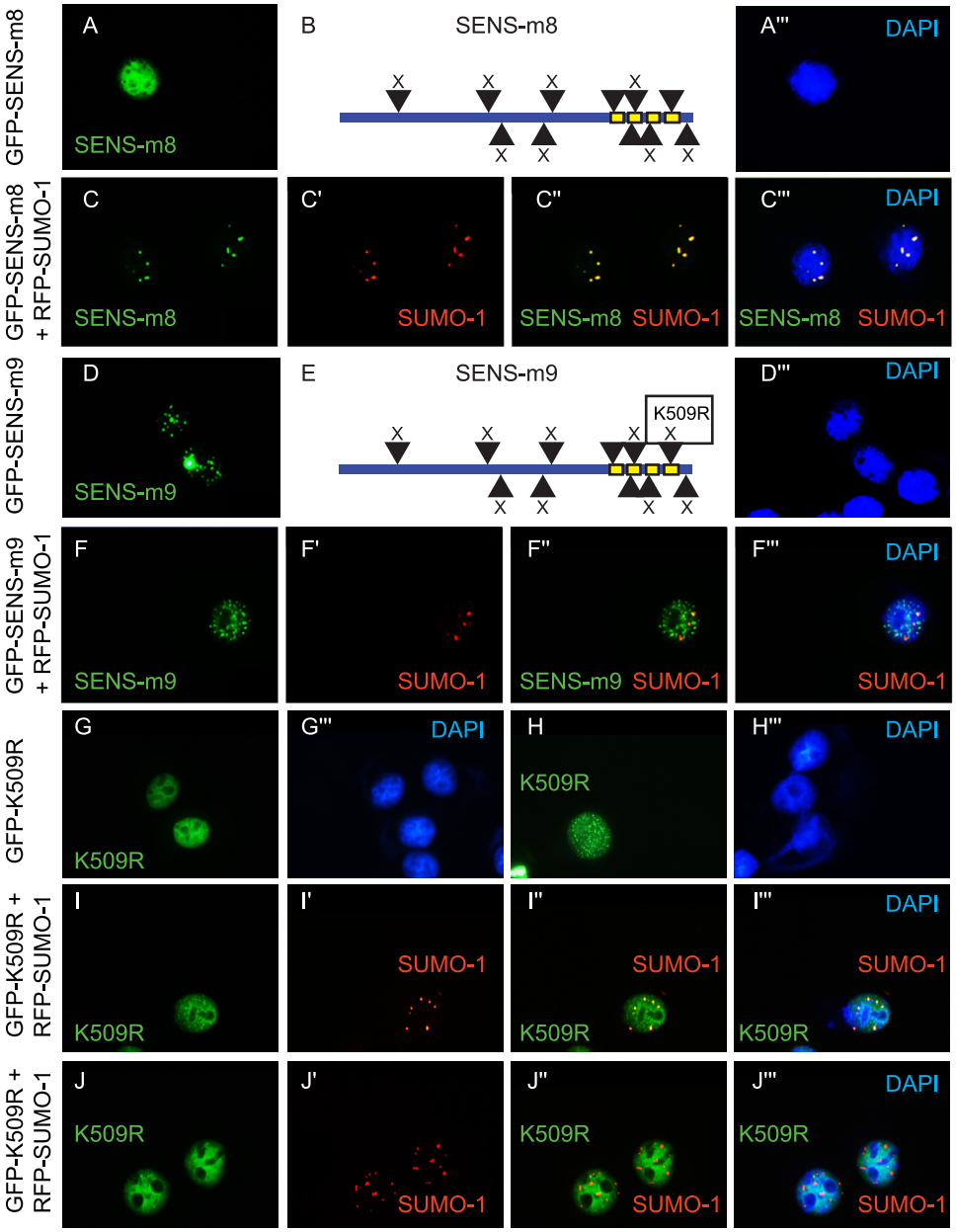

FIG 4 Mutation of SUMOylation motifs in SENS reveals K509 is important for SUMO-dependent SENS relocalization in HeLa cells. (A to C) Mutation of 8 of the 11 SUMOylation motifs in SENS (SENS-m8 in schematic [B]) had no effect on its localization. (A) SENS-m8 showed a diffuse nuclear distribution similar to that of wild-type SENS (Fig. 3) (C) In the presence of SUMO-1, SENS-m8 relocalized to the PML bodies in a fashion similar to that of wild-type SENS. (D to F) Mutation of one extra lysine (K509R) in SENS-m9 (schematic [E]) affected SUMO-dependent relocalization. (D) SENS-m9 showed punctate nuclear distribution, which does not appear to correspond to the PML bodies. (F) In the presence of SUMO-1 the SENS-m9 protein remained in a wider distribution pattern than was seen for SUMO-1. (G and H) The single-site mutant $SENS^{K509R}$ showed two different patterns of distribution in the absence of SUMO. The first pattern (G) was indistinguishable from that of the wild type (compare with Fig. 3A; found in 40.8% ± 2.7% of transfected cells), while the second (H) showed the $SENS^{K509R}$ localized in dots, unlike the pattern seen for the wild type but more diffuse than for SENS-m9 (59.3% ± 2.7% of transfected cells). (I and J) Upon cotransfection with SUMO-1, relocalization of $SENS^{K509R}$ occurred in a small proportion of cells (data not shown; 4.7% ± 1.2% of cells), but in most cells localization was defective, i.e., diffuse with nuclear dots, 53.97% ± 1.73% of transfected cells (I); and diffuse localization, 41.3% ± 0.7% of transfected cells (J). In each case the letter alone shows the GFP-SENS variant (green); a letter followed by one prime shows the RFP-SUMO-1 (red), a letter followed by a double prime shows red/green overlay; and a letter followed by a triple prime labels the red/green/blue overlay (blue, DAPI [4′,6-diamidino-2-phenylindole]), except in those cases where SENS variants are transfected alone, and then the triple prime indicates DAPI alone.

seen for other SUMO substrates subject to SUMOylation at unidentified sites (14, 54). The presence of alternative SUMOylation sites is also supported by the yeast two-hybrid interaction data. However, the strong effect of K509 motif mutations on SENS colocalization with SUMO in HeLa cells suggests that this site may be of major importance *in vivo*.

SENS SUMOylation mutants are refractory to SUMO stimulation of proneural/SENS transcriptional activation. We tested

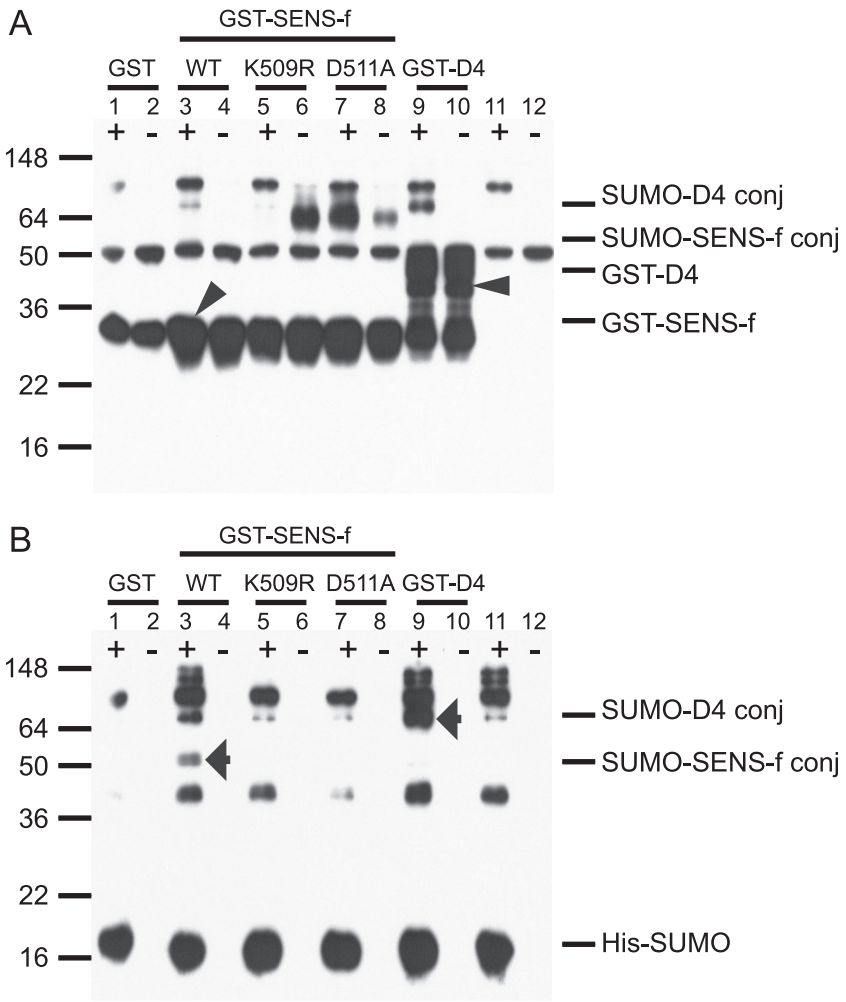

FIG 5 (A, B) *In vitro* SUMO conjugation assays demonstrate the importance of K509 and D511 residues for SUMOylation. *In vitro* SUMO conjugation assays were carried out according to the method described in reference 12 for N-terminally GST-tagged 20-amino-acid SENS fragments (GST-SENS-f) corresponding to amino acids 500 to 519 of the full-length SENS, for the wild-type K509R and D511A SENS variants, with GST-D4* (DAXX-4 fragment) as a positive control. The reactions were run on 12.5% SDS-PAGE and then subjected to Western blotting. (A) SUMOylation substrates (arrowheads) were visualized using anti-GST antibodies. (B) SUMO conjugates were visualized by probing the stripped membrane from panel A with anti-His antibodies. SUMO conjugates were observed only for the SENS wild-type fragment (lane 3) and the DAXX-4 fragment (lane 9) and not in lanes 5 and 7 (GST-SENS-f-K509R and GST-SENS-f-D511A, respectively). A + or − symbol above each lane indicates the presence or absence, respectively, of His-SUMO.

the effect of the K509R and D511A SUMO motif mutations on proneural/SENS activity in the S2 cell reporter gene assay (Fig. 6). In contrast to wild-type SENS, the activities of both mutated versions were unresponsive to UBC9/SUMO cotransfection (Fig. 6B, bars 8 and 10 compared to bar 6). These data are consistent with K509 being an important SUMOylation site. Furthermore, they suggest that SUMO modification of SENS at K509 promotes proneural/SENS synergy. Our next step was to assess whether SUMOylation of SENS is important *in vivo*.

Overexpression of SUMO enhances proneural/SENS-induced neurogenesis *in vivo*. In *Drosophila*, ectopic expression of proneural proteins or SENS induces the formation of supernumerary sensory organs (31, 47). To assess whether SUMOylation of SENS plays a role *in vivo*, we targeted the expression of SENS and SC in the wing imaginal discs, using an SOP/PNC GAL4 driver [109(2)68Gal4]. Misexpression of SENS in wing imaginal discs using 109(68)Gal4 results in ectopic external sense organ (bristle) production due to the specification of supernumerary SOPs (Fig. 7A). Misexpression of SUMO using the same Gal4 driver did not affect bristle number (Fig. 7A). However, SUMO misexpression was able to enhance the bristle number increase due to SENS misexpression (Fig. 7A). Interestingly, SUMO misexpression also enhanced the ability of proneural proteins to promote ectopic sense organs. SUMO enhanced the production of ectopic bristles in response to SC misexpression (Fig. 7B). We suggest that the effect of SUMO on SC function occurs via modification of endogenous SENS. Significantly, SUMO also enhances the effect of ATO misexpression (Fig. 7C). ATO misexpression results in a reduction in bristle number due to respecification of the SOPs to give internal chordotonal sense organs (31). Simultaneous misexpression of SUMO enhanced this reduction by ATO. This suggests that SUMO does not promote bristle formation *per se* but instead promotes neurogenesis via a specific effect on proneural/SENS activity. It is notable that SUMO overexpression alone has no effect on endogenous neurogenesis, suggesting that

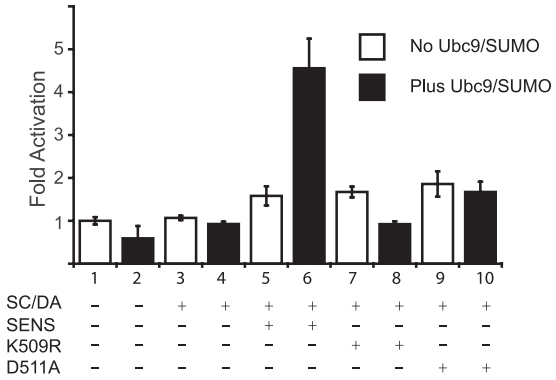

FIG 6 SENS SUMOylation motif mutants are refractory to SUMO stimulation. Transcriptional activation of the *ac* enhancer reporter gene construct pGL4-AcE-luc by SC/DA heterodimer is slightly elevated by addition of wild-type SENS (column 5 versus column 3). This SENS/SC/DA interaction is strongly and specifically elevated upon coexpression of SUMO and UBC9 results (column 6 versus column 5). In contrast, the SUMO motif mutants $SENS^{K509R}$ and $SENS^{D511A}$ were unresponsive to the addition of SUMO and UBC9 (columns 8 versus 7 and 10 versus 9).

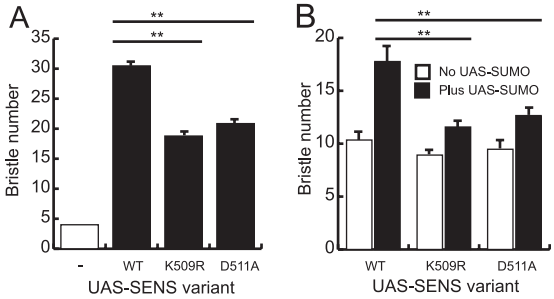

FIG 8 SENS proteins with SUMOylation motif mutations promote fewer ectopic bristles than the wild type and show reduced genetic interaction with SUMO *in vivo*. The 109(2)68 GAL4 line was used to drive ectopic expression of SENS and SENS variants ($SENS^{K509R}$ and $SENS^{D511A}$) in the absence or presence of overexpressed SUMO, and scutellar bristles were counted. (A) Misexpression of SENS SUMOylation motif mutant proteins resulted in fewer ectopic bristles than were observed for misexpression of wild-type SENS. Crosses were incubated at 25°C. (B) SENS SUMOylation motif mutant proteins had reduced susceptibility to the synergistic effect of comisexpressed SUMO protein (black bars). Crosses were incubated at 21°C in order to reduce GAL4 activity and hence prevent saturation of the ectopic bristle phenotype upon comisexpression of SUMO and SENS. One-way analysis of variance (ANOVA) followed by a *post hoc* Tukey test showed significant differences between wild-type and mutant protein phenotypes as indicated by the double asterisks ($P < 0.01$).

SUMO becomes limiting only under the circumstances of proneural/SENS misexpression.

The effect of the SUMOylation motif mutants on SENS's promotion of ectopic bristles was assessed. Transgenic lines were constructed with SENS misexpression constructs inserted into an attP2 landing site on chromosome 3L (10). Insertion at the same landing site allowed quantitative comparison of their effects on neurogenesis (Fig. 8A and B). The 109(2)68Gal4 line was used to drive ectopic expression of SENS or SENS variants ($SENS^{K509R}$ and $SENS^{D511A}$) (Fig. 8A). The two mutated SENS proteins promoted significantly lower numbers of ectopic bristles than did wild-type protein. The effects of the mutations on the *in vivo* interaction between SUMO and SENS were also assessed (Fig. 8B). In this experiment we exploited the temperature dependence of GAL4 protein production (17) to prevent saturation of the bristle phenotype, by reducing the temperature of the crosses. Under these conditions, the differences between the SENS variants alone were not apparent (Fig. 8B, white bars) but the synergistic effect of SUMO overexpression was still observed (Fig. 8B, black bars). Under these conditions, the mutant SENS proteins were less susceptible than wild-type SENS to the synergistic effect of SUMO overexpression (Fig. 8B, black bars). The ratio of bristle number for the SENS/SUMO misexpression combination compared with that for SENS alone was 1.7 for the wild type and 1.3 for both the $SENS^{K509R}$ and $SENS^{D511A}$ mutants. These results indicate that the K509 SUMOylation site in SENS is important for most of the genetic interaction with SUMO. The residual presence of interaction is consistent with the suggestion of additional alternative SUMOylation sites within SENS. However, another likely explanation could be the fact that endogenous (therefore wild-type) SENS contributes to ectopic neurogenesis induced by UAS-SENS. Overall, the results are consistent with K509 being important for SUMO enhancement of SENS activity but do not rule out the possible importance of other lysines as targets too.

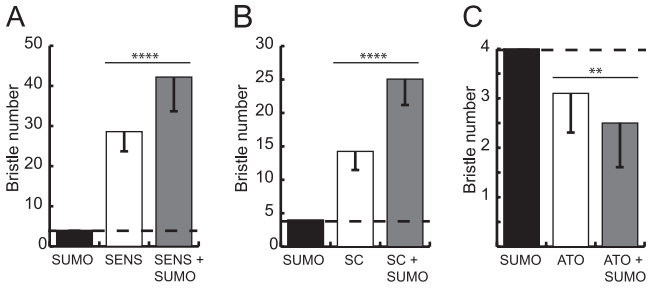

FIG 7 Overexpression of SUMO enhances proneural-SENS-induced neurogenesis *in vivo*. The SOP/proneural cluster driver, 109(2)68Gal4, was used to drive expression of neural proteins either alone or in combination with UAS-SUMO at 25°C (A and B) and 18°C (C), and the scutellar bristles were counted. (A) Effect of UAS-SUMO on UAS-SENS phenotype. UAS-SENS promoted ectopic bristles on the scutellum (for the wild type, the number is four, indicated by a dashed line). Overexpression of SUMO alone did not affect the bristle number, but it enhanced the effect of UAS-SENS. (B) UAS-SUMO similarly enhanced the ectopic bristle phenotype driven by UAS-SC. (C) Effect of UAS-SUMO on UAS-ATO phenotype. In contrast to SENS and SC, misexpressed ATO results in a decrease in bristle number from the wild-type number due to respecification of the SOPs to give internal chordotonal sense organs. Simultaneous misexpression of ATO and SUMO enhanced this reduction. The effect of SUMO in each case was statistically significant as assessed by the Wilcoxon test. ****, $P < 0.0001$; **, $P < 0.01$.

DISCUSSION

SUMO enhances SENS's ability to promote proneural activity in reporter gene assays and to promote neurogenesis *in vivo*. Our data suggest that SUMO modification promotes proneural gene autoregulation and is also likely to be important in the regulation of downstream proneural target genes. SUMOylation has a positive effect and deSUMOylation a negative effect on transcriptional activation by proneural/DA/SENS ternary complexes in S2 cells. In contrast, no effect on proneural protein activity was observed in the absence of SENS, suggesting that SENS is the target for SUMO. This is supported by the interactions between SUMO and SENS in the HeLa cell relocalization and yeast two-hybrid assays, the direct covalent interaction between SENS and SUMO detected in S2 cells

and the *in vitro* SUMOylation assay, and the effect of mutating a putative SUMOylation motif in SENS Zn finger 4.

The last assay identified a lysine (K509) in the fourth Zn finger as a candidate for a major SUMOylation site in the SENS sequence. Mutation of this lysine to arginine (K509R) resulted in disruption of SUMO-dependent SENS interaction in the HeLa cell assay, a SENS protein refractory to SUMO stimulation in the S2 cell transcriptional assay, and reduced genetic interaction between SENS and SUMO *in vivo*. Furthermore, evidence from yeast two-hybrid assays and from analysis of S2 lysates for $SENS^{K509R}$ suggested that additional unidentified lysines may also be SUMOylated. Interestingly, the basal transcriptional synergy between SENS and proneural/DA heterodimers observed in S2 cells appears to be largely dependent on endogenous SUMOylation, as the synergy is strongly reduced by ULP1 cotransfection. Consistent with this, proteomic analysis has shown that S2 cells express SUMO, UBC9, and UBA2 (SAE1) proteins (24).

SUMO affects the activity of the proneural/DA/SENS ternary complex. While our evidence suggests that SENS is the target of SUMOylation, we do not rule out the possibility that the other proteins of the complex may also be SUMOylated, but at present there is no evidence for this. Notably, the ATO sequence has no ΨKxD/E motifs, while SC has been shown to be unaffected by SUMOylation in a separate study (1). DA has three potential SUMOylation motifs, but mutation of each of these does not affect proneural/DA/SENS synergy (our unpublished observations).

Our evidence suggests that SUMOylation of SENS enhances transcriptional synergy via an effect on the proneural/SENS ternary complex itself. How might SENS SUMOylation mediate this increase in transcriptional synergy? SUMOylation can exert a positive effect on transcriptional activation by various mechanisms including alteration of subcellular localization and mediation of interaction with transcriptional coactivators or DNA (40, 61, 64). In the present case, we suggest that either (i) SUMOylation increases the affinity of SENS for the proneural protein heterodimer, hence favoring formation of the more transcriptionally active ternary complex; (ii) SUMOylation increases the transcriptional activation or DNA-binding ability of the ternary complex, perhaps by inducing a conformational change; or (iii) SUMO simply stabilizes SENS. SUMO is known to modulate protein-protein interactions in other systems: for example, SUMOylation of RanGAP1 promotes binding of RanB2 either by creating or exposing a binding site (46), while nuclear magnetic resonance (NMR) studies have provided direct evidence of a SUMOylation-induced conformational change in thymine-DNA glycosylase (56). The identified SUMO site of SENS (K509) is within the fourth Zn finger. This is significant because the Zn finger has been shown to be unimportant for DNA binding by SENS but contributes to the transcriptional synergy mediated by proneural/SENS interaction (3). It is conceivable therefore that SUMOylation at this site increases the affinity of SENS for proneural/Da heterodimers. This would be similar to the proposed enhanced interaction between the TEA family transcription factor Scalloped and its coactivator Vestigial upon SUMOylation of the latter (59).

A major effect of SENS (and therefore SUMO) in promoting SOP specification appears to be via promoting proneural/DA activation of autoregulatory enhancers. This proneural/SENS autoregulatory synergy is thought to have an important role in bypassing the negative regulatory effects of the Hairy/E(SPL) (HES) bHLH repressor proteins downstream of Notch signaling (29). It

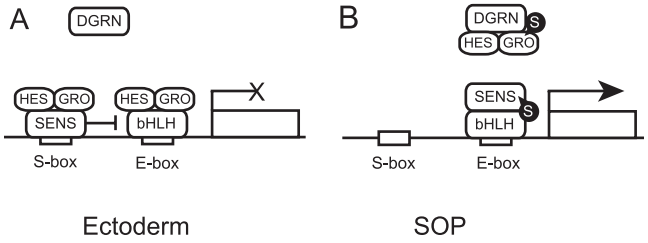

FIG 9 A model for the role of SUMO in SOP specification. This model combines our investigation of SENS with recent studies of HES-GROUCHO (1, 5). (A) In the ectodermal cells adjacent to the SOP, SENS acts as a DNA-binding-dependent repressor of proneural gene autoactivation by proneural-DA heterodimers (bHLH) (29). An additional level of proneural gene repression results from E(SPL)-GROUCHO (HES-GRO) corepressor complexes binding to both SENS and the bHLH heterodimer (3). (B) In the SOP, SUMOylation (S) of SENS promotes SENS-proneural transcriptional activation of proneural gene expression. Also in the SOP, SUMOylation of GRO promotes its binding by the ubiquitin ligase DGRN, an association that disrupts the association of GRO and E(SPL), leading to derepression (1, 5). The S-box is the SENS binding site and the E-box is the proneural-DA heterodimer binding site. In this panel the S-box is shown unoccupied, but it is not known whether SUMOylation of SENS prevents its DNA binding.

is therefore interesting that another role for SUMOylation in SOP specification has recently been identified in relation to HES repressors. A model has been proposed in which the repressive activity of HES proteins during neurogenesis (as well as segmentation and sex determination) is disrupted by the SUMO-targeted ubiquitin ligase (STUbL) Degringolade (DGRN) (1, 2, 5). DGRN binds to SUMOylated Groucho (GRO), the corepressor of HES. This interaction, as well as ubiquitination of the HES proteins, is thought to disrupt the HES-GRO interaction, leading to increased neurogenesis. Hence, these two SUMO-dependent mechanisms (i.e., increased SENS coactivation and decreased HES repression) may work in a complementary manner to enhance neurogenesis (Fig. 9). It will be important to determine how SOP-specific SUMOylation is regulated in order to elucidate the developmental mechanisms involved.

As well as acting as a proneural coactivator, SENS directly represses some target genes via binding to S box motifs. It is therefore conceivable that SUMO can relieve SENS repression of its targets by its promotion of ternary proneural-SENS formation, effectively sequestering SENS from binding to its target S boxes. For example, SENS directly represses the SOP-specific gene *rhomboid* (*rho*), the activation of which is crucial for the epidermal growth factor receptor (EGFR)-dependent recruitment of secondary SOPs during neurogenesis (25, 39, 65, 69). ATO indirectly activates *rho* expression in larval abdominal SOPs by binding SENS and preventing it from binding and repressing the *rho* enhancer (65). If, for example, SUMOylation enhances SENS binding to ATO, then it may play a role in activation of *rho* and other direct targets of SENS repression.

SENS belongs to the GPS (Gfi1/Pag-3/SENS) family of proteins (30), and its mammalian orthologues are the oncogenes Gfi1 and Gfi1b. The Gfi proteins differ from SENS in containing transcriptional repression SNAG domains near their N termini, and Gfi1 and Gfi1b have been reported to act mainly as transcriptional repressors (22, 71). Despite these differences, in the mammalian peripheral nervous system Gfi1 functions in close connection to proneural factors. For example, it works in concert with Atoh1 (the mammalian homologue of ATO) in the specification of inner

ear hair cells (35, 48, 63, 67). Gfi1 also has a crucial role in the formation of retinal ganglion cells in the mammalian eye, working downstream of a different ATO homologue, Atoh7 (63, 66). Gfi1 also has key developmental roles in the lung and intestine, working together with the mammalian AC/SC homologue Ascl1 in pulmonary neuroendocrine cell production (34) and with Atoh1 in the production of secretory cells of the intestine (55).

It has been suggested that Gfi1 and the mammalian proneural proteins may act as transcriptional coactivators in a way similar to that of the *Drosophila* proteins (34, 55, 63), although direct evidence for this is lacking. If corroborated, such interactions could conceivably be modulated by SUMO in a mechanism similar to that which we have found in *Drosophila*. Interestingly, this is supported by the observation that, like SENS, Gfi1 associates with SUMO pathway proteins including the SUMO-conjugating enzyme UBC9 in a yeast two-hybrid assay (16), although no other evidence has so far been reported for SUMOylation of Gfi1. While SENS has four C-terminal Zn fingers, Gfi has six. The sixth Zn finger of Gfi1 is not needed for DNA binding and is equivalent to the fourth Zn finger of SENS. This is the location of the putative SUMOylated lysine (K509) in SENS, which is completely conserved in the context of the SUMOylation motif in Zn finger 6 of Gfi1. In conclusion, it is possible that Gfi1 activity is modulated by SUMOylation, and this could have an effect via a molecular mechanism similar to that which we have identified for SENS.

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